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14. ABSTRACT Loss of nerve function occurs when a nerve is severed. Any significant loss of nerve tissue requires a graft to restore continuity and promote nerve regeneration and recovery of function. Presently, there is no acceptable nerve grafting method for the repair of extensive nerve damage. An acellular nerve allograft product is available for the reconstruction of short to moderate length nerve defects, however, in its present form the allograft is not suitable for the repair of lengthy nerve defects. The aims of this project are to evaluate several long (7cm) nerve allograft technologies. All experimental tasks scheduled for years 1 and 2 were completed. A 6-month no-cost extension was approved to complete the extensive data analyses and documentation. An overview of findings indicates that the SIS-graft provided for nerve regeneration and meaningful recovering of nerve function that, in several cases was better than autografting. Other decellularized allografts tested did not perform well in this repair model. Reports of these outcomes for publication are in progress.					
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INTRODUCTION

Loss of nerve function is complete when a nerve is severed and significant recovery without surgical intervention is rare. Any significant loss of nerve tissue requires a graft to restore continuity and promote nerve regeneration. Presently, there is no acceptable nerve grafting method for the repair of extensive nerve damage. Recently, an acellular nerve allograft product was brought to market that is now used widely for the reconstruction of short to moderate length nerve defects. However, this allograft, in its present form, is not suitable for the repair of lengthy nerve defects, like those often encountered with battlefield trauma. This proposal aims to greatly expand the application of nerve allografts for the reconstruction of extensive nerve damage. One approach to apply an acellular allograft in the repair of long nerve defects is the stepping-stone graft. Stepping-stone grafts combine two moderate length allografts with a small, interposed autologous nerve segment. A second approach is to apply a biodegradable gel coating to protect and prolong the efficacy of the acellular graft once implanted.

Specific Aims are to: 1) Evaluate 7 cm acellular allograft prototypes 4 weeks after implantation in rabbits. Key aspects of graft viability and integration will be assessed to finalize grafting protocols. 2) Conduct a 26-week preclinical evaluation of 7 cm stepping-stone allografts (SS-grafts) and 7 cm hydrogel-coated allografts in the reconstruction and rehabilitation of extensive peripheral nerve injury in rabbits. Control conditions include a standard acellular allograft and cellular autograft (the gold standard in nerve grafting). Outcomes will be evaluated by translational neurological testing.

BODY

Task 1. Evaluate nerve grafts in 4-week study (months 1-6):

Task 1a. Collect, process (decellularize) and prepare 7 cm acellular allografts (n=16)(8 rabbits)(months 1-3).

- i) Evaluate detergent decellularization, including extraction of chondroitin sulfate chains (CS56 immunolabeling), myelin (sudan black staining), axons (neurofilament immunolabeling), and DNA (propidium iodide staining).
- ii) Evaluate integrity of decellularized grafts, including retention of nerve sheaths (laminin immunolabeling) and CSPG core proteins (CSPG neo-epitope immunolabeling).
- iii) Test methods for thorough coating of grafts with hydrogel (spiked with dye).

Progress:

Task 1a was completed successfully. Nerves were collected from donor rabbits processed by three detergent decellularization schemes. We evaluated the outcome of the three processing methods by several histological techniques. These included hematoxylin and eosin, sudan black, Hoescht, laminin, neurofilament, S-100, CS56 and CS-6. Results of each method were semi-quantitatively scored (0=Not extracted, 1=Partially extracted, 2=Mostly extracted, 3=Fully extracted and R= Redistributed).

Results for three detergent processing schemes (DC1, DC2, DC3) are shown in Figures 1-8. Based on these evaluations a final allograft processing protocol was established. The DC2 protocol rendered rabbit nerve similar to the nerves processed by the Avance® method (DC1). Rabbit peroneal nerve allografts (n=18, including 2 backups) were processed by protocol DC2. Nerve trims were evaluated as describe above and all criteria for nerve graft processing were met.

Next, various hydrogel types, concentrations and application methods were tested on nerve segments in vitro. The hydrogel product DuraSeal was most effective using the concentration and instructions provided by the manufacturer for use in other tissues. A trough (mold) was developed to aid application and retention of the hydrogel around the nerve until it polymerized. An optimal volume of hydrogel per cm of nerve was established.

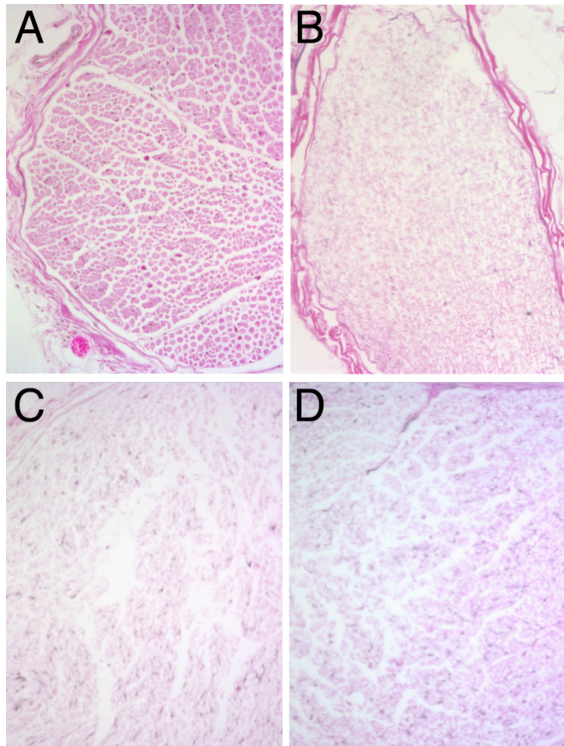


Figure 1. H & E Staining.

<u>Panel</u>	<u>Score</u>
A. Normal nerve control	0
B. DC1	2
C. DC2	2
D. DC3	2

H&E staining for general histology showed the three processing methods achieved extensive cellular extraction and retention of overall nerve sheath integrity.

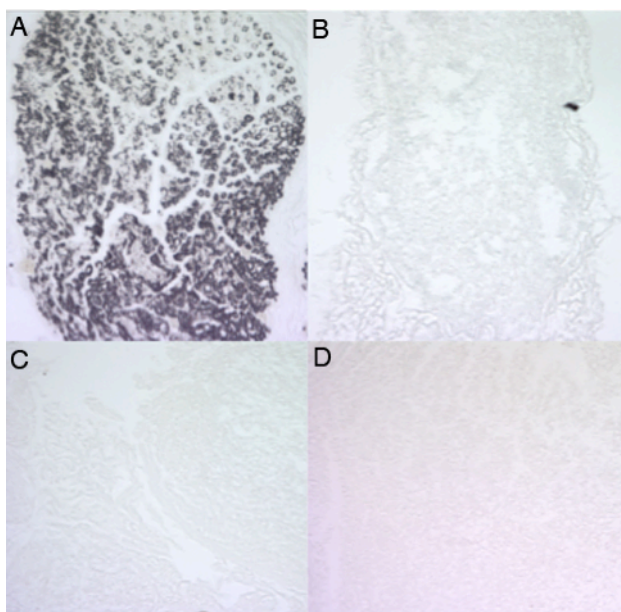


Figure 2. Sudan Black Staining.

<u>Panel</u>	<u>Score</u>
A. Normal nerve control	0
B. DC1	3
C. DC2	3
D. DC3	3

Sudan black staining for myelin showed the three processing methods to be equally and highly effective at extracting myelin.

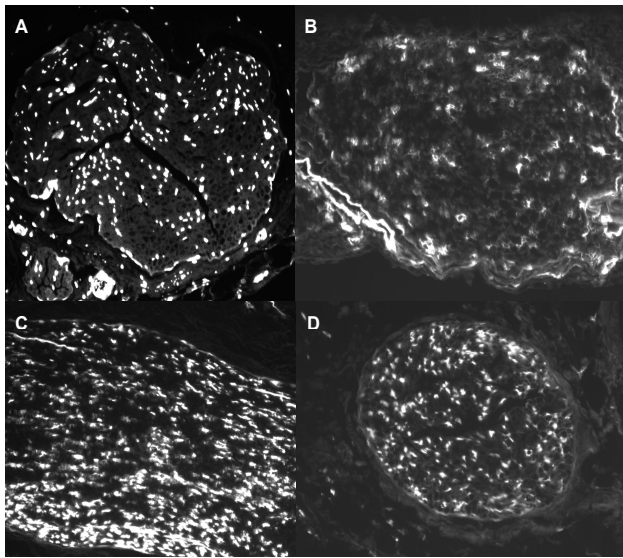


Figure 3. Hoescht Staining.

<u>Panel</u>	<u>Score</u>
A. Normal nerve control	0
B. DC1	2R
C. DC2	1R
D. DC3	1R

Hoescht staining for DNA showed the three processing methods did not eliminate DNA but rather dispersed and redistributed chromosomes.

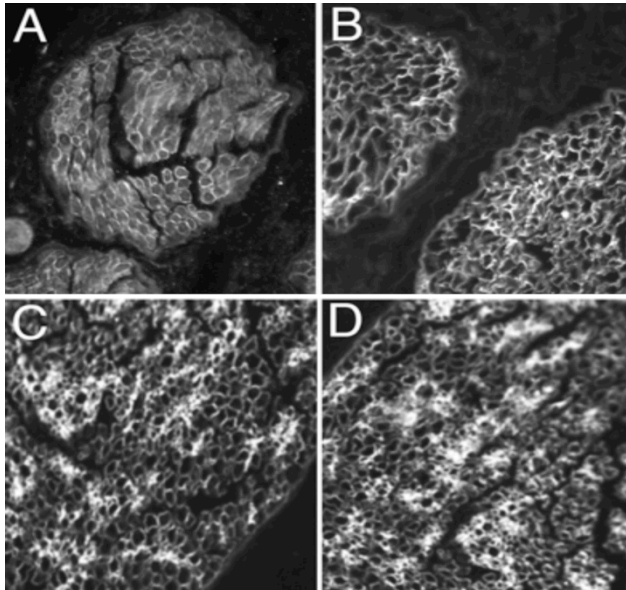


Figure 4. Laminin Immunostaining.

<u>Panel</u>	<u>Score</u>
A. Normal nerve control	0
B. DC1	2R
C. DC2	1R
D. DC3	1R

Laminin is a major component of the nerve sheaths including the important basal lamina surrounding axons. Laminin immunolabeling showed the three processing methods had an equal effect on the preservation of basal lamina structure.

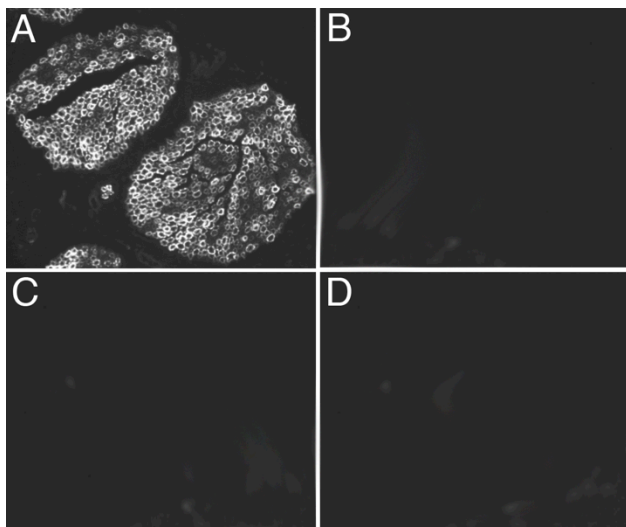


Figure 5. S-100 Immunostaining.

<u>Panel</u>	<u>Score</u>
A. Normal nerve control	0
B. DC1	3
C. DC2	3
D. DC3	3

S-100 immunolabeling (a cytoplasmic protein in Schwann cells) showed the three processing methods equally and very effectively extracted the cytoplasm of Schwann cells.

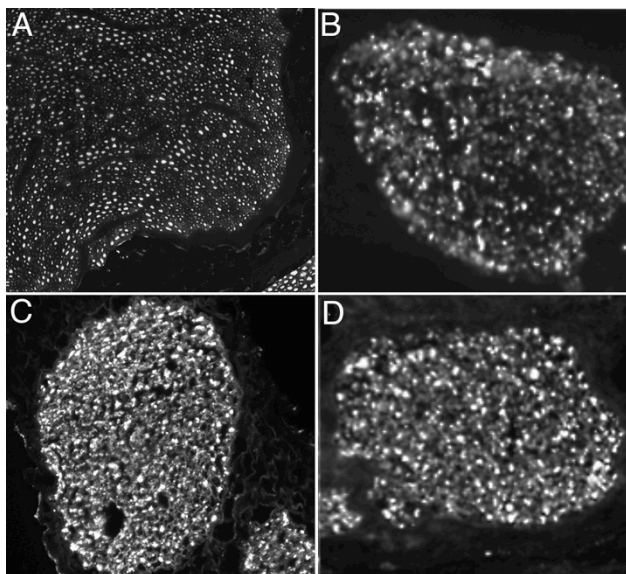


Figure 6. NAP-4 Neurofilament Staining.

<u>Panel</u>	<u>Score</u>
A. Normal nerve control	0
B. DC1	1R
C. DC2	1R
D. DC3	1R

Neurofilament immunolabeling (a marker of the neuronal/axonal cytoskeleton) showed the three processing methods only partially extracted and redistributed the axonal cytoskeleton.

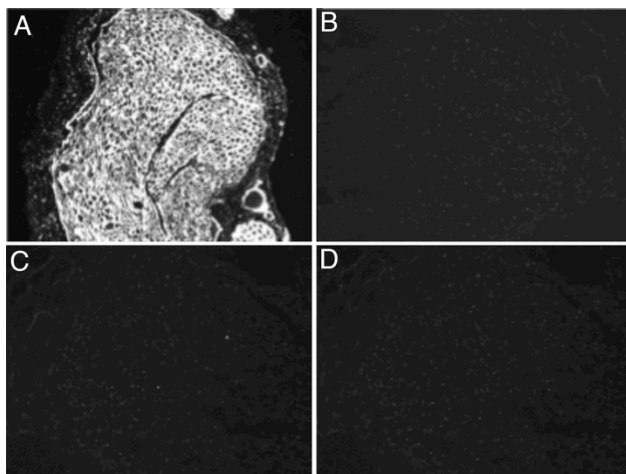


Figure 7. CS56 Immunostaining.

<u>Panel</u>	<u>Score</u>
A. Normal nerve control	0
B. DC1	3
C. DC2	3
D. DC3	3

CS56 antibody labels the side-chains of chondroitin sulfate proteoglycans. All processing methods include the same treatment step with chondroitinase ABC, an enzyme that degrades the side-chains. CS56 immunolabeling showed the three processing methods effectively eliminate the chondroitin sulfate side-chains after detergent extractions.

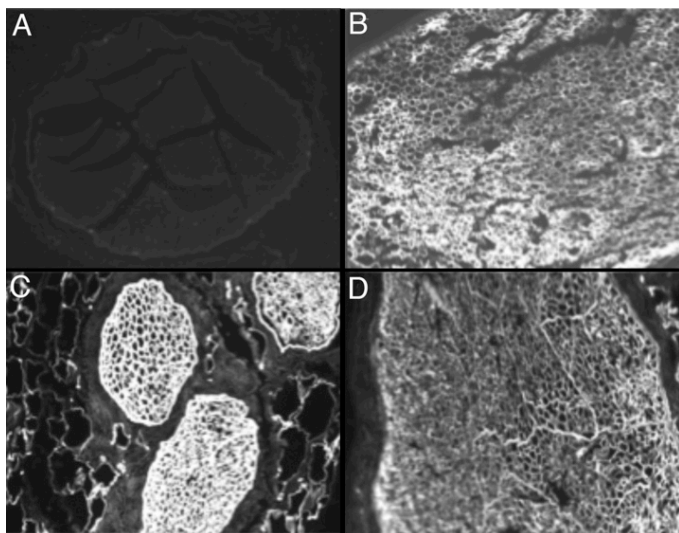


Figure 8. CS-6 Neopeptide Immunostaining.

<u>Panel</u>	<u>Score</u>
A. Normal nerve control	0
B. DC1	3
C. DC2	3
D. DC3	3

CS-6 neopeptide immunolabeling reveals proteoglycan core protein that was stripped of CS-6 side-chains by chondroitinase ABC treatment. Positive C6-6 immunolabeling showed the three processing methods effectively eliminate the chondroitin sulfate side-chains and yet the core protein remains intact in the basal lamina.

Task 1b. Implant nerve allografts in rabbits (16 rabbits) (months 2-4).

- i) Perform nerve grafting with control (undivided, uncoated) DCI-grafts (n=4).
- ii) Perform nerve grafting with DCI-grafts coated with hydrogel (n=4).
- iii) Perform nerve grafting with SS-grafts in the 3-1-3 configuration (n=4).
- iv) Perform nerve grafting with SS-grafts in the 4-1-2 configuration (n=4).

Progress

Implantation of the four nerve allograft types was completed on schedule and without complications. All animal hosts survived and remained in good health throughout the 4-week survival period.

Task 1c. Evaluate outcomes of nerve grafting after 4 weeks, including:

- i) Axon morphometrics (scoring neurofilament immunopositive axons)
- ii) Graft integrity, cellularity and viability within the graft (H&E staining)
- iii) Schwann cell migration from autologous nerve segment (S100 immunolabeling).

Progress

Task 1C was completed and the goals of the 1-month grafting study were achieved. All graft hosts were terminated and the engrafted nerves removed. Nerves were processed, sectioned and histology and immunocytochemistry was performed. Axon morphometrics indicated nerve regeneration occurred and advanced well into all of the graft types. As expected, because the proximal aspect of all grafts was the same, no significant differences were found in initial nerve regeneration or proximal graft integrity (see Fig. 9).

Differences in the distal grafts (not yet occupied by regenerating nerve) were readily observed, as expected. Based on our observations the following conclusions were made:

1) DCI-grafts (control) were integrated into the recipient nerve and appeared to be immunotolerated well. As expected, the onset of vacuolization and deterioration was observed in the distal aspect of the grafts.

2) The outcome of DCI-grafts coated with hydrogel was complex and unclear. The hydrogel evoked an unusual inflammatory response. Furthermore, there was considerable variation in the hydrogel coating that in some instances failed to remain intact in vivo. These findings indicated the hydrogel coating condition was problematic, required further development and, therefore, was unsuitable at this point for our long-term grafting study. In an earlier project we performed

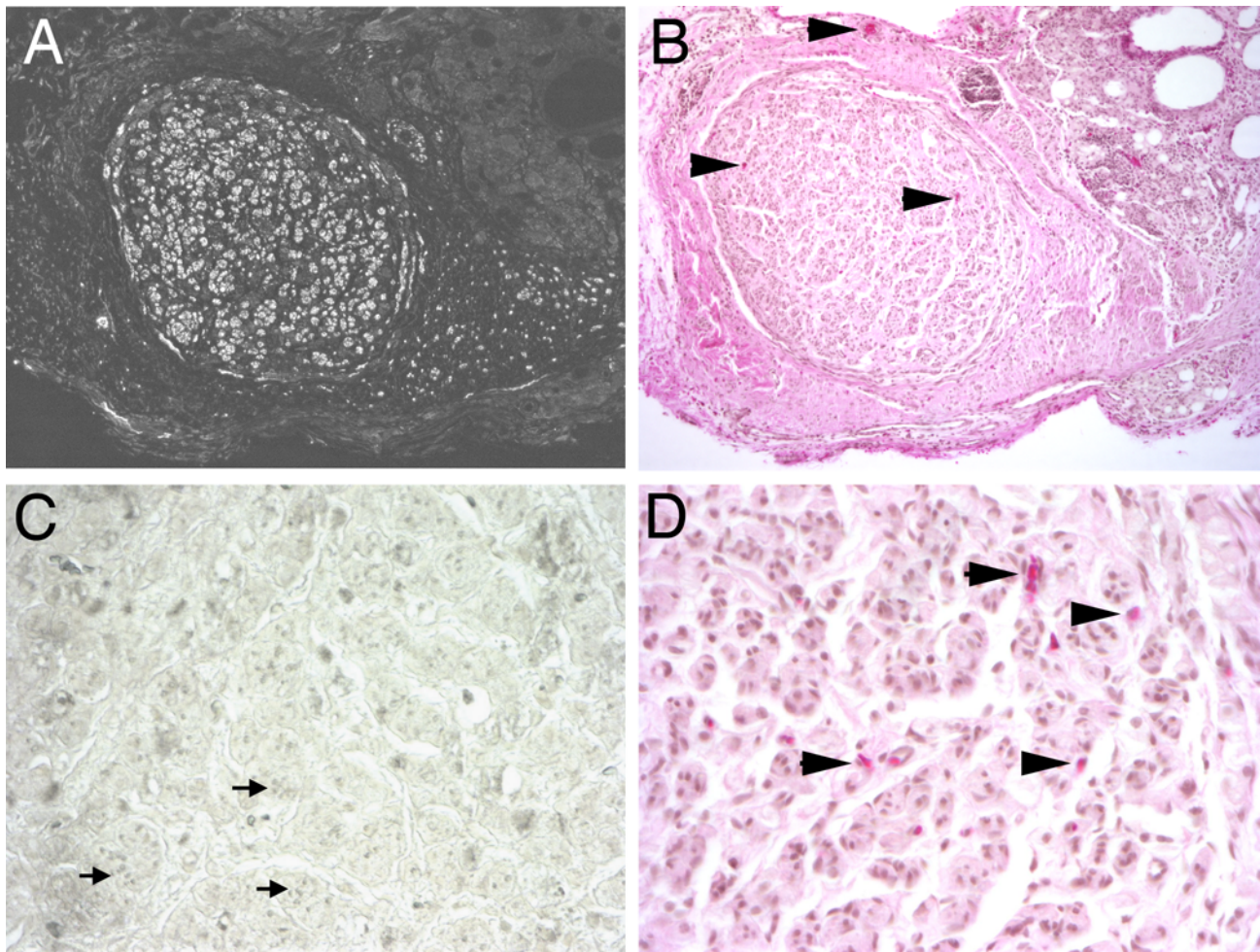


Figure 9. Representative photomicrographs of the proximal DCI-graft component, 4 weeks post-implantation.

- A: Neurofilament immunolabeling for axons showed extensive nerve regeneration into the grafts.
 B: H&E staining indicated grafts were integrated and immunotolerated. (Arrowheads: blood vessels)
 C: Sudan black stained clusters of myelinated axons (arrows) indicating nerve maturation within the graft.
 D: H&E staining (high mag of B) showed extensive blood filled capillary network within the grafts (arrowheads).

preliminary testing using a very different approach to protect the DCI-grafts in vivo. This involved placing the distal half of the graft inside a biodegradable, natural biologic tube. This tube is a multi-laminar extracellular matrix from small intestine submucosa (SIS), marketed as the AxoGuard® Nerve Protector (AxoGen Corp). This FDA-approved material is used in numerous clinical applications. Applied to nerve, the SIS tube initially prevents the influx of inflammatory cells and protects the nerve graft from degradation. Importantly, the SIS tube is eventually remodeled to an epineurial sheath-like structure. The time course of protection and remodeling seems ideally suited for use with long nerve grafts. Also, the tubes are preformed, eliminating the variable encountered with application of the hydrogel. The tube will be placed around the distal half of the nerve graft to protect it for several months, while allowing normal early cell repopulation and nerve regeneration within the proximal part of the nerve graft (not protected by the tube). This modification to our animal use protocol was approved by our IACUC and then by ACURO.

3) All SS-grafts well immunotolerated well. One SS-graft lack continuity with the proximal recipient nerve (the sutures did not hold) and was excluded from the evaluation. SS-grafts contained viable Schwann cells migrating from the live autograft segment into the flanking DCI graft segments,

as hoped. The SS-grafts with a 4-1-2 configuration (compared to the 3-1-3) showed more extensive Schwann cell population, especially toward the distal end of the graft. This outcome is optimal and the 4-1-2 configuration was selected for further testing in long-term grafting.

Based on these outcomes, graft constructs and configurations for long-term study were established (see Task 2b).

Task 2. Evaluate nerve grafts in 26-week study (months 7-24):

Task 2a. Collect, process and prepare 7 cm acellular allografts (18 rabbits)(months 7-10).

Progress

Thirty-eight peroneal nerves were collected from 19 donor rabbits. The grafts were decellularized and processed by the protocol established in Task 1. The grafts were then frozen, packaged and shipped to a contract facility for gamma irradiation sterilization. After irradiation, the return shipment was severely damaged in transport by a commercial service. The grafts had thawed and the sterile packaging was breached rendering the grafts usable for in vivo grafting. A modification to the IACUC protocol was submitted to allow for replacement donor animals. The protocol modification was approved by our IACUC and then by ACURO. Replacement rabbits were obtained. The nerves were harvested and processed successfully. Test samples met the histological criteria established in Task 1 and passed in vitro sterility tests. Despite having to repeat the collection and production of nerve grafts we remained on schedule for long-term grafting experiments. This Task was completed successfully.

Task 2b. Implant nerve grafts in rabbits (48 rabbits) (months 8-14).

Progress

The implantation of the four nerve graft types was completed ahead of schedule. As stated above, the hydrogel coated nerve graft condition was replaced with the SIS tube encased nerve graft. Also, two SS-graft configurations were evaluated in Task. The SS-grafts with 4-1-2 configuration had more extensive Schwann cell population, especially toward the distal end of the graft. This outcome is optimal and the 4-1-2 configuration was selected for further testing in long-term grafting. The revised design is shown in Table 1.

Table 1. 26-week evaluation of nerve graft technologies (revised)

Nerve graft (7 cm)	Sample	End-point	Evaluations
Autograft	n=12	26 weeks	<ul style="list-style-type: none"> • Toe-spread function • Trans-graft electrophysiology • Axon morphometrics • Graft integrity, cellularity, viability
DCI-graft	n=12		
DCI-graft + SIS tube	n=12		
SS-graft (4-1-2)	n=12		

All nerve grafting surgeries were performed successfully. All graft recipients tolerated the surgery without complications and remain in good health. As anticipated, some recipients have a propensity to chew their foot due to the imposed loss of sensory nerve function. This transient condition is monitored closely and managed proactively by the application of gauze wraps and bitter-tasting spray. Thus far, no detrimental self-mutilation has occurred. Animals are monitored daily and health concerns addressed. To date, no animals have met terminated criteria or have been eliminated from the protocol.

Task 2c. Assess progressive recovery of function after nerve repair (months 12-20).

- i) Beginning 16 weeks after nerve grafting, perform weekly function testing and collect video documentation of all rabbits (n=48).
- ii) Extract still frames (n=4) from each test session.
- iii) Perform image analysis to measure toe-spread function and calculate index for each test session.
- iv) Plot and perform statistical analyses of function data.

Progress:

A rabbit's ability to spread its toes is lost after transection of the peroneal nerve. In this study transected peroneal nerves were repaired with various nerve graft constructs. The loss of toe-spread function persists until the cut nerve fibers regrow through the graft and distal nerve and reinnervate the muscles that control movements of the toes. The methods used to test toe-spread function are described in Figure 1.

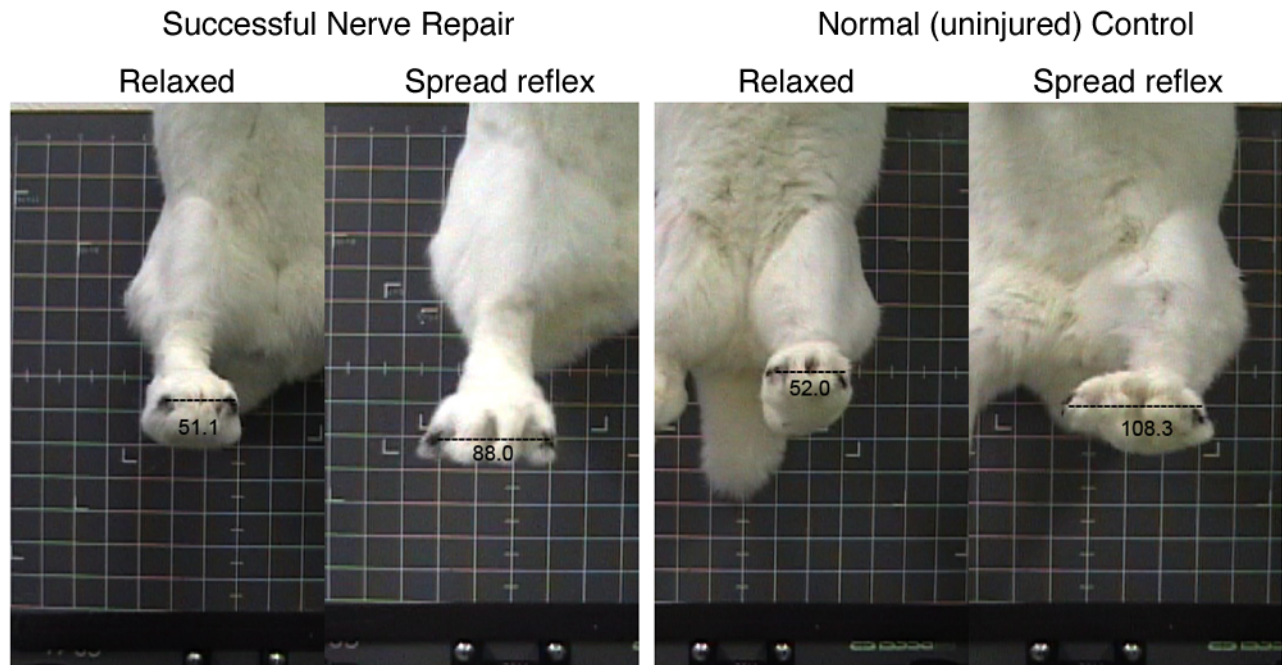


Figure 1. Measuring the toe-spread reflex in rabbit. The rabbit was lifted by the tuft of the neck. When fully relaxed, the rabbit was suddenly lowered a few inches to simulate the feeling of falling and induce the toe-spread reflex. The toe-spread behavior was recorded by digital video photography and still frame images were captured from 6-8 trials per test session. The distances from digits II-V were measured by image analysis software. The Toe-spread Index (TSI) was calculated as the mean of the Spread measurements divided by the mean of the Relaxed measurements. A TSI indicative of no function was 0.9-1.1. A normal TSI was 1.5-2.1. Shown above are measurements after a highly successful nerve graft reconstruction (left panels; TSI, $88.0 \div 51.1 = 1.72$) and a normal rabbit response (right panels; TSI, $108.3 \div 52.0 = 2.08$).

Task 2c was completed. All animals were assessed weekly until termination 26 weeks after receiving the nerve graft. Videos were taken and numerous still photos (single frames) were extracted. We have completed calculating the toe spread function scores at all time points. After termination the engrafted nerves were examined for nerve-graft continuity. Animals with a loss of continuity due to failure of sutures at the coaptation were excluded from the analyses. Based on this criterion, two or three animals were excluded from each group.

Outcomes of recovery of function are shown in Table 2. The autograft condition gave the best recovery of function with 9/10 animals displaying some degree of toe-spread. Five of nine rabbits in the DCI-graft condition showed some degree of toe-spread function recovery. Seven of nine rabbits in the SS-graft condition showed toe-spread. No recovery of function was observed for animals in the DCI-graft+SIS tube condition. The mean TSI score for all the graft conditions were well below normal (1.5-2.1). The highest individual TSI score was 1.4 and there were more individual animals with high scores in the SS-graft condition than in the other groups.

Table 2. Recovery of toe-spread function 26 weeks after nerve grafting

Graft condition	Animals with TS function	Mean TSI score
Autograft	9/10	1.27
DCI-graft	5/9	1.25
DCI-graft + SIS tube	0/10	1.09
SS-graft (4-1-2)	7/9	1.24

The Toe-spread Index (TSI) was calculated as described in Figure 1.

2d. Perform trans-graft electrophysiology 26 weeks after grafting (months 14-20).

- Obtain single stimulus-generated compound action potentials through the implant at stimulation intensities for large diameter myelinated A-fibers and small diameter myelinated and unmyelinated fibers.
- Measure conduction latency and velocity for each fiber group.
- Obtain paired stimulus compound potentials and determine alterations in conduction through the graft.
- Evaluate progression of incomplete regeneration by stimulation at different sites within and distal to the graft.

Progress

Immediately prior to termination of the animal subjects, the nerve grafts were exposed surgically and trans-graft electrophysiology performed to determine the conduction of signals through the grafted nerve. All electrophysiological recordings were performed. The recordings were analyzed and data extracted to determine nerve conduction velocities and the extent of muscle reinnervation. Findings are shown in Table 3.

Table 3. Trans-graft nerve conduction 26 weeks after nerve grafting

Graft condition	Animals with conduction	Mean conduction (m/sec)
Autograft	9/10	34.9
DCI-graft	2/9	8.7
DCI-graft + SIS tube	0/10	0.0
SS-graft (4-1-2)	4/9	13.6

Electrophysiological recordings were best in the autograft condition and 9/10 animals had meaningful conduction of action potential. Only 2/9 rabbits in the DCI-graft condition and 4/9 rabbits in the SS-graft condition met criteria for measurable conduction. None of the animals in the DCI-graft+SIS tube condition showed trans-graft conduction. The best individual conduction

velocities were observed in the autograft condition (37.2 - 54.9 m/sec) and were mostly half that recorded for normal nerves (mean= 100.4 m/sec).

Task 2e. Perform histological/immunocytochemical examination on grafts (months 19-27).

- i) Axon morphometrics (scoring neurofilament immunopositive axons)
- ii) Graft integrity, cellularity and viability within the graft (H&E staining)
- iii) Schwann cell migration from autologous nerve segment (S100 immunolabeling).

Task 2f. Perform histomorphometric scoring for nerve regeneration (months 23-29).

Task 2g. Analyze and document results (months 24-30).

Progress

All rabbits survived the 26-week survival period in good health. All animals were terminated, the engrafted nerves were removed and processed for histological and immunocytochemical examinations. Key points in the nerves were sectioned and stained. Image analysis to score regenerated axons that grew through the grafts and into the host nerve (indicative of successful nerve repair) was completed. A summary of results is shown in Table 4.

Table 4. Trans-graft axonal growth 26 weeks after nerve grafting

Graft condition	Animals with distal axons	Mean axon count (labeled pixels)
Autograft	9/10	1,129,171
DCI-graft	4/9	219,220
DCI-graft + SIS tube	0/10	0
SS-graft (4-1-2)	7/9	410,588

Scoring axonal regeneration at other points (e.g., within the grafts) was also completed to determine the temporal progression of nerve regeneration. Other features of nerve growth within the grafts have been examined. Representative observations are shown in Figure 2. Nerve regeneration in some graft conditions was poor. Numerous additional tissue sections of the graft content were examined to better understand the dynamics of nerve regeneration within the grafts. Representative observations of a failed nerve graft are shown in Figure 3. Deterioration of the distal aspect of the graft was a conspicuous feature of failure. Successful processed allografts were thoroughly compared to the most successful autographs. Representative evaluations are shown in Figure 4. Detailed analyses of these data are ongoing as we prepare written reports of our findings.

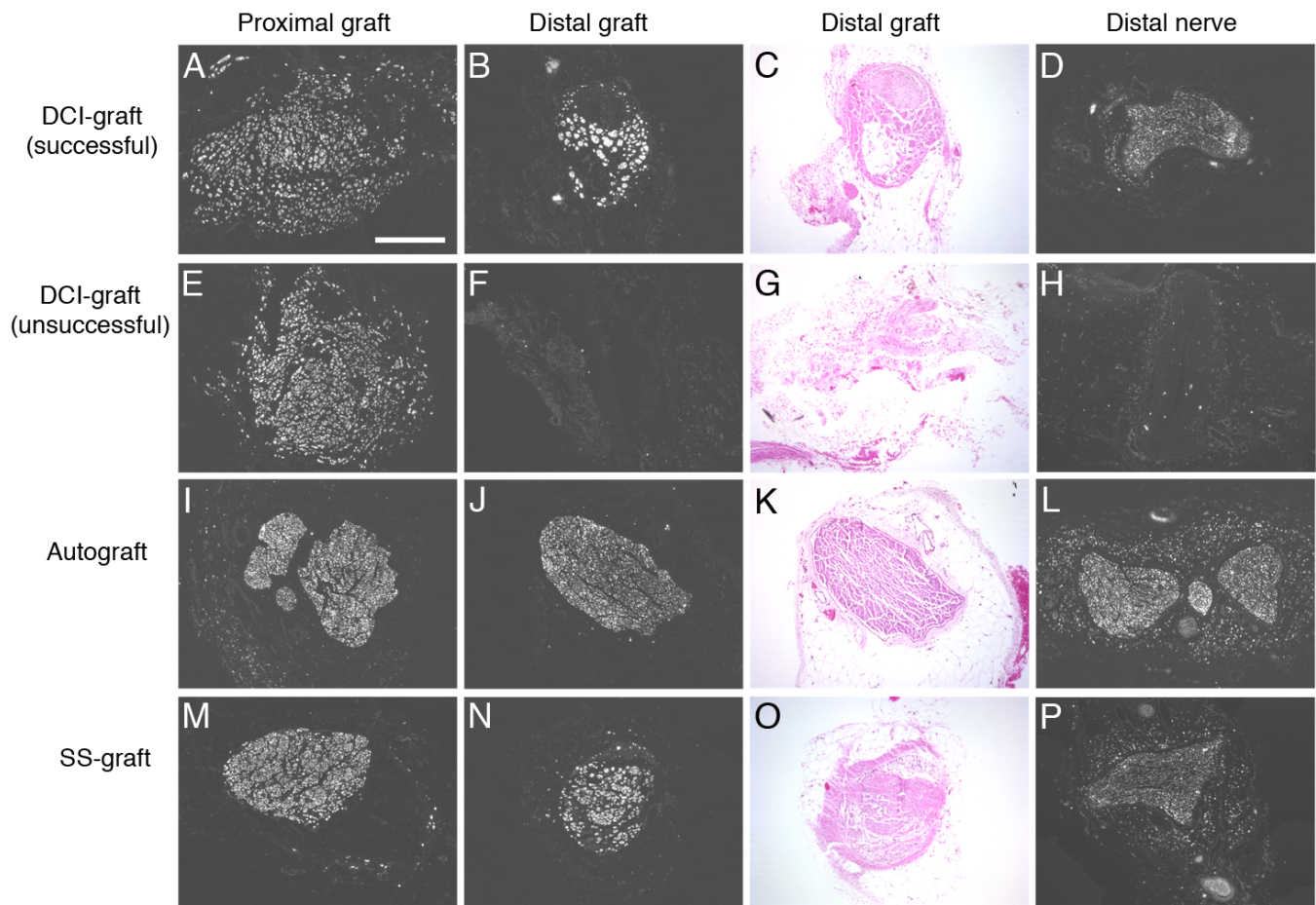


Figure 2. Regeneration within and distal to 7 cm grafts at 26 weeks post-implantation.

A: Successful Processed Allograft, neurofilament immunolabeled axons at 0.5 cm into the proximal graft
 B: Successful Processed Allograft, neurofilament immunolabeled axons at 1.5 cm proximal to the distal coaptation
 C: Successful Processed Allograft, H&E stain at 1.5 cm proximal to the distal coaptation (as in B)
 D: Successful Processed Allograft, neurofilament immunolabeled axons at 0.5 cm into the distal nerve

E: Failed Processed Allograft, neurofilament immunolabeled axons at 0.5 cm into the proximal graft
 F: Failed Processed Allograft, neurofilament immunolabeled axons at 1.5 cm proximal to the distal coaptation
 G: Failed Processed Allograft, H&E stain at 1.5 cm proximal to the distal coaptation (as in F)
 H: Failed Processed Allograft, neurofilament immunolabeled axons at 0.5 cm into the distal nerve

I: Autograft, neurofilament immunolabeled axons at 0.5 cm into the proximal graft
 J: Autograft, neurofilament immunolabeled axons at 1.5 cm proximal to the distal coaptation
 K: Autograft, H&E stain at 1.5 cm proximal to the distal coaptation (as in J)
 L: Autograft, neurofilament immunolabeled axons at 0.5 cm into the distal nerve

M: Acellular Allograft, neurofilament immunolabeled axons at 0.5 cm into the proximal graft
 N: Acellular Allograft, neurofilament immunolabeled axons at 1.5 cm proximal to the distal coaptation
 O: Acellular Allograft, H&E stain at 1.5 cm proximal to the distal coaptation (as in N)
 P: Acellular Allograft, neurofilament immunolabeled axons at 0.5 cm into the distal nerve
 Scale bar A-P, 360 μ m

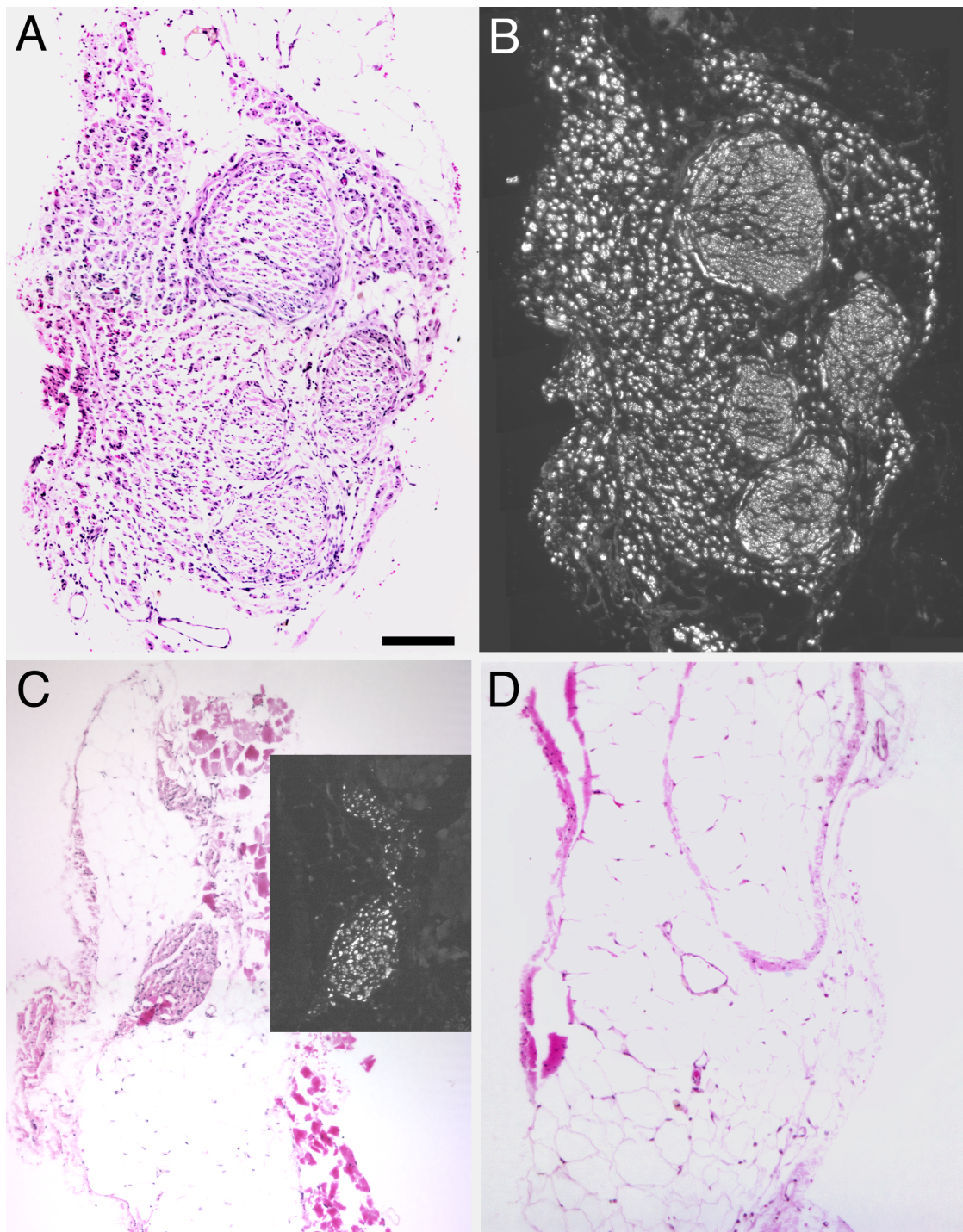


Figure 3. Failed regeneration within 7 cm rabbit nerve Processed Allografts at 26 weeks post-implantation.

A: Proximal graft, H&E stain at 0.5 cm into the graft.

B: Proximal graft, neurofilament immunolabeled axons at 0.5 cm into the proximal graft

C: Mid-graft, H&E stain at 2.5 cm into the proximal graft. Inset, neurofilament immunolabeling.

D: Distal graft, H&E stain at 1.5 cm from the distal coaptation.

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Processed Allograft

Autograft

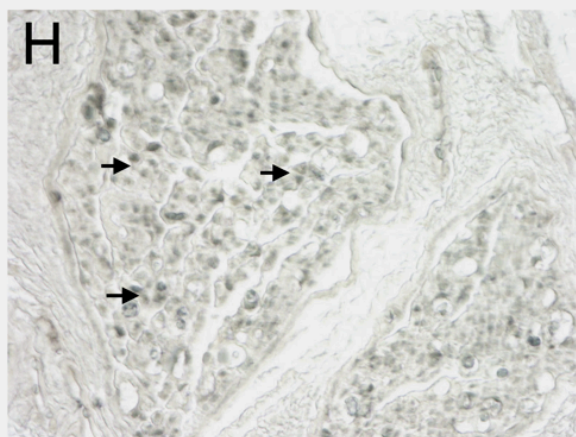
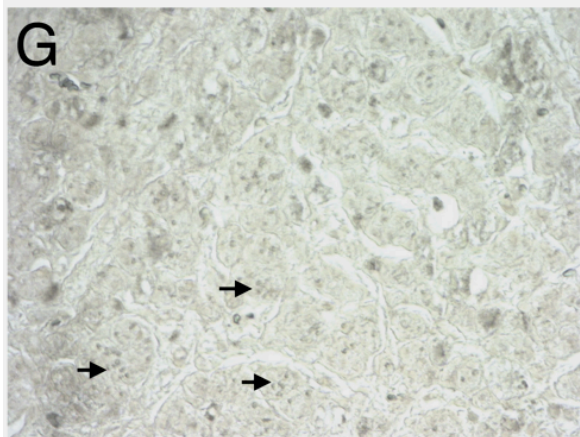
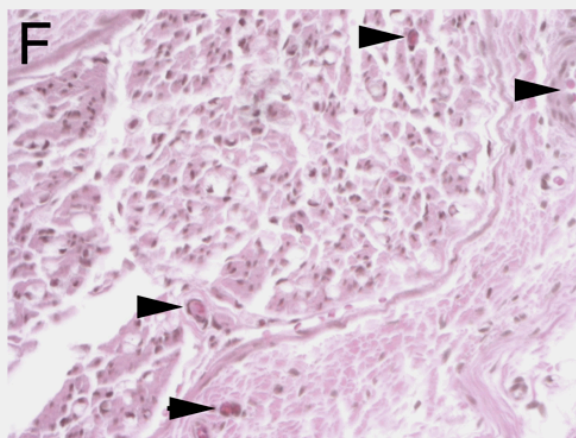
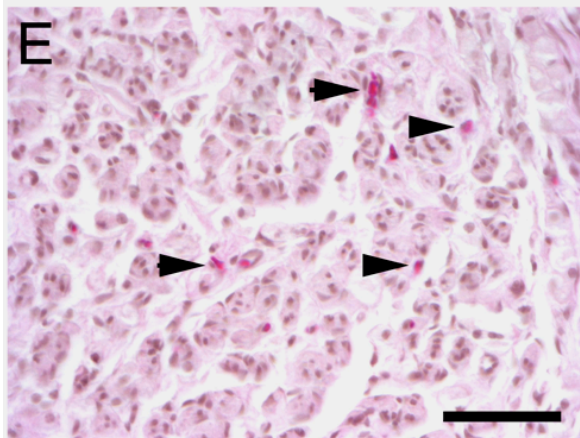
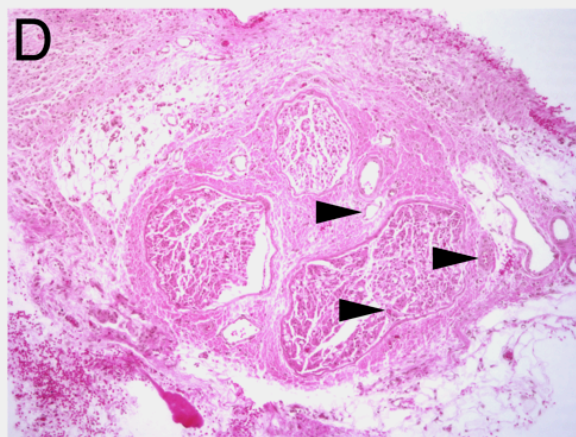
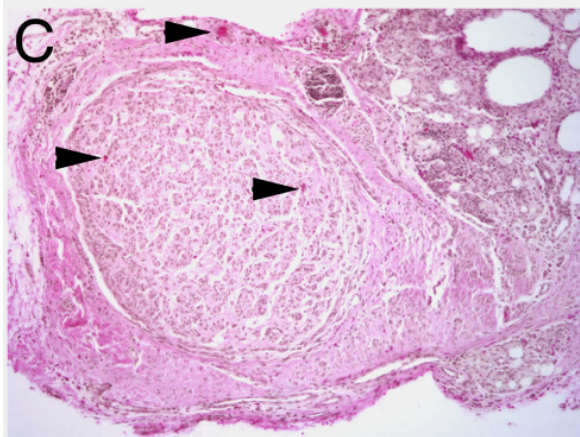
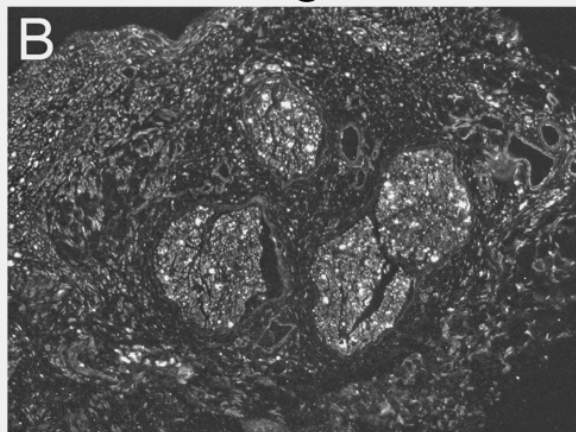
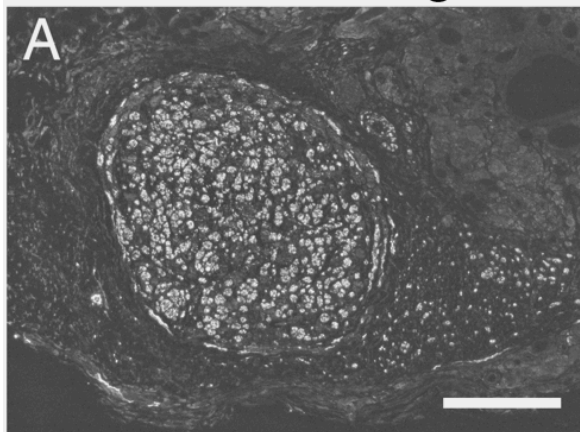


Figure 4. Representative photomicrographs of the proximal aspect of 7 cm grafts at 4 weeks post-implantation.

A: Processed Allograft, neurofilament immunolabeled axons.
B: Autograft, neurofilament immunolabeled axons.
C: Processed Allograft, H&E stain. Arrowheads: blood vessels.
D: Autograft, H&E stain. Arrowheads: blood vessels.
E: Processed Allograft, H&E stain. Arrowheads: blood vessels.
F: Autograft, H&E stain. Arrowheads: blood vessels.
G: Processed Allograft, Sudan black stain. Arrowheads: clusters of myelinated axons.
H: Autograft, Sudan black stain. Arrowheads: clusters of myelinated axons.

Scale bar A-D, 360 μ m

Scale bar E-H, 90 μ m

KEY RESEARCH ACCOMPLISHMENTS:

- A nerve graft processing protocol was established for rabbit peripheral nerve that mimics the production of the human nerve allograft product (Avance®) used clinically. This includes detergent decellularization, treatment with chondroitinase ABC, and irradiation sterilization.
- A long stepping-stone graft (with a live autograft segment interposed between two DCI-graft segments) was established.
- A nerve allograft model in rabbit was established to test graft variables and outcomes for the repair of extensive nerve damage using long nerve grafts.
- Forty-eight rabbits received nerve grafts, survived the recovery period in good health and nerve regeneration was evaluated successfully.
- Determined that the DCI grafts were marginally effective.
- Determined an autograft insert into a DCI graft (the SS-graft) improved the outcome significantly.
- Determined that an SIS tube around the distal aspect of a DCI graft was detrimental and all grafts in this condition failed.

REPORTABLE OUTCOMES: None

CONCLUSION: The Tasks completed in years 1 and 2 established a novel decellularized nerve allograft model in rabbit. Several nerve allograft prototypes were examined and protocols established for long-term in vivo evaluation. The goal is to discover ways to increase the effective length of decellularized nerve allografts. Presently nerve allografting is limited to the repair of moderate (1-4 cm) nerve gaps. This project tested four grafting technologies involving 7 cm nerve allografts in a small diameter rabbit nerve model. All nerve grafting procedures and initial evaluations were completed on schedule. Analyses of the extensive data collected are ongoing and a 6-month no-cost extension was approved for this purpose. An overview of the data indicates that the SIS-graft (a hybrid of decellularized and autograft segments) provided significant recovery of function that, in several cases, was better than autografting. With proper technique the SIS-graft may offer an effective alternative to conventional autografting using off-the-shelf nerve that overcome the length limitations of conventional nerve allografting.